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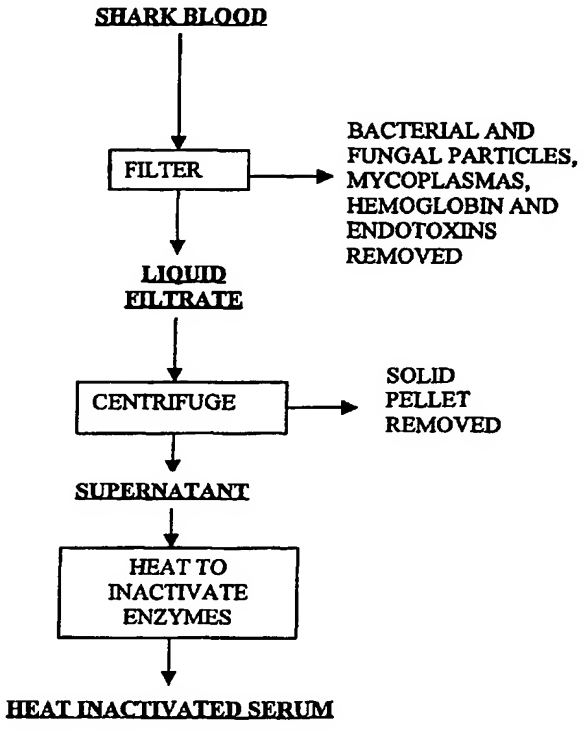

(57) Abstract: A new anti-angiogenic and anti-inflammatory extract derived from the cartilage of embryonic cartilaginous fish, and a method for producing the extract has been developed. The extract is derived by culturing the chondrocytes from shark or shark embryo. Additionally, a specialized growth media containing serum from the blood of a cartilaginous fish has been developed for culturing the chondrocytes to produce the extract.

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METHOD OF PREPARING ANTI-ANGIOGENIC DRUG FROM CARTILAGE AND CHONDROCYTES AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/426,258, filed November 13, 2002, the contents of which hereby incorporated by reference into the present disclosure.

FIELD OF THE INVENTION

The present invention relates to a method of preparing an anti-angiogenic drug from the cartilage and chondrocytes of shark.

BACKGROUND

Angiogenesis is a fundamental process by which new blood vessels are formed. It is an essential step in many physiological events, such as reproduction, development and wound healing. Under normal conditions, angiogenesis is highly regulated. However, many diseases are driven by persistent unregulated angiogenesis. Such diseases and conditions include cancer, solid tumors, rheumatoid arthritis, osteoarthritis, psoriasis, diabetic retinopathy, age related macular degeneration, restinosis, Kaposi's sarcoma, leukemia, metastasis, benign tumors, Osler-Webber syndrome, myocardial angiogenesis, plaque neovascularization, telangeictasia, hemophilic joints, angiofibroma, wound granulation, glaucoma, corneal graft rejection, atherosclerosis, scleroderma, keloids, inflammation, and fetal conception.

For example, tumor growth and metastasis are angiogenesis-dependent. Most primary solid tumors go through a prolonged state of avascularization, and apparently dormant growth in which the maximum size attainable is 1-2 mm in diameter. Up to this size, tumor cells can obtain the necessary oxygen and nutrient by simple passive diffusion. These microscopic tumor masses eventually "switch on" angiogenesis by

recruiting surrounding mature host blood vessels to begin sprouting new blood vessel and capillaries which grow toward, and eventually infiltrate the tumor mass. This sets in motion the potential for relentless expansion of tumor mass and hematogenous metastasis spread. In rheumatoid arthritis, new capillary blood vessels invade the joints and destroy the cartilage. In diabetic retinopathy, new capillaries form in the retina and invade the vitreous, bleed, and eventually cause blindness (Folkman, (1995) Nature Med. 1(1):27-31; Folkman, and Klagsbrun (1987) Science 235:442-447; Walsh, D.A. (1998) Rheumatology 38(2):103-112; Healy et al., (1998) Hum. Reprod. Update 4(5):736-396).

Inhibitors of Angiogenesis and Tumor therapy

Tumor growth can be limited or prevented by blocking tumor angiogenesis, particularly by directly targeting the vascular endothelial cells of newly formed, immature blood vessels. This approach prevents any new expansion of the tumor mass and eventually cause sustained regressions of established solid tumors to a size of approximately 1-2 mm in diameter where survival is possible without a blood vessel supply. Such a therapeutic approach is designated as "dormancy-inducing" and meant to control the disease in a chronic fashion.

Published literature describes a broad range of medical benefits from shark cartilage including angiogenic properties. Shark cartilage extracts have also been developed and marketed as dietary supplements. There also has been criticism of shark cartilage as an effective treatment for advanced cancer (see Miller et. al., (1998) J. Clin. Oncol. 16:3649-3655).

Carl A. Luer, of Mote Marine Laboratory in Sarasota, Fla., identified a group of three proteins recovered from shark cartilage which are believed to be suitable for retarding or reversing tumor growth without the harmful side effects of radiation (Los Angeles Times, Aug. 27, 1990, pp B3).

DuPont (U.S. Pat. No. 6,028,118), and Balassa (U.S. Pat. No. 4,822,607) both describe an extract of shark cartilage having anti-angiogenic and anti-inflammatory properties, as well as the method of extraction and their use in treatment. Neither the composition nor the molecular nature of these anti-angiogenic components of both of these patents have been characterized or described. Both DuPont and Balassa extract directly from shark cartilage which is a finite and non-renewable resource. Their

resultant product is a large "soup" of active components, either in solid or liquid extract form, rather then isolated specific compound.

Liang reports isolating an extract of shark cartilage, which unlike the protein extracts of Luer, Gruber, DuPont and Balassa, is believed to be a proteoglycan (See "Isolation, Fractionation, and Characterization of Angiogenesis Inhibitor from Shark Cartilage" Jeannie Liang (1997) (student thesis) California State University, Fresno, CA). The extract was tested for angiogenic properties using an endothelial cell assay. Liang extracts directly from shark cartilage by a process which involves coring the centrum of the shark cartilage with a steel punch tool, lyophilizing the cored disks, pulverizing the disks in a blender, and extracting preferably with water (see Liang (1997) supra, pp.9 and 34).

However, sharks are a limited resource and difficult to obtain in large quantities. As such, it is desirable to culture shark chondrocyte cells so that the cells can be re-cultured and increased as much as desired.

Limited work has been done on this by Grogan and Lund, who cultured immunocytes from sharks and rays (Gorgan and Lund (1990) "A culture system for the maintenance and proliferation of shark and sting ray immunocytes. J. Fish Bio. **36**:633-642). Additionally, Hartman, et al. attempted to culture a number of shark tissues, but not cartilage (Hartman J., et al. (1992) "Routine establishment primary elasmobranch cell cultures," In Vitro Cell. Dev. Biol. **28A**:77-79). These attempts were unsuccessful in that the cells from the tissues that did show some initial growth could not be passes and subsequently died (see "An Angiogenesis Inhibitor from Cultured Shark Chondrocytes", O'Hara C. (1995) (student thesis) California State University, Fresno, CA, p.12).

O'Hara and Wong have reported isolating an angiogenesis inhibitor from cultured chondrocytes of adult shark cartilage and from the culture media (see O'Hara,(1995), supra, and O'Hara and Wong (1996) (student thesis) "Cultured Shark Chondrocytes Secrete an Angiogenesis Inhibitor" California State University, Fresno, CA). The cells cultured according to the study, however, had very slow growth rates since the chondrocytes were unable to attach to the walls of the tissue culture by themselves. (See IN VITRO, Journal of the Society of In Vitro Biology (1996) **32**:37A reporting on the O'Hara and Wong publication and O'Hara (1995) supra. In order to promote attachment of the chondrocytes, an artificial matrix was used. This

increased growth; but not to practical densities (see O'Hara (1995) supra; O'Hara and Wong (1996) supra, and INVITRO (1996) supra). According to these studies, "Shark chondrocyte cultures may produce the inhibitory factor, but to date, no successful in vitro cultivation of shark chondrocytes has been reported." O'Hara and Wong (1996) INVITRO, supra.

Furthermore, the cartilage of adult sharks is very brittle and physically hard. It therefore must be dissociated to release the chondrocytes before culturing. The procedure for dissociation requires exposing the cartilage to a solution containing collagenase until dissociation occurs, and then centrifuging to separate the chondrocytes from the solution (see O'Hara (1995) supra and O'Hara and Wong (1996) supra). This procedure risks breaking down the integrity of the chondrocytes and increases the risk of contamination.

The O'Hara (1995) thesis suggests using an embryonic shark as a chondrocyte source, because the cells would be less differentiated. However, O'Hara fails to suggest any procedure for doing this, or any results of changing the starting material.

DESCRIPTION OF THE INVENTION

The present invention provides a new anti-angiogenic and anti-inflammatory extract and method of producing the extract. The extract is isolated from cultured chondrocytes derived from the cartilage of a cartilaginous fish, e.g., a shark, and from the chondrocyte culture media. Additionally, a specialized growth media has been developed for producing the new extract. The extract comprises the biologically active agent which comprises a proteoglycan or glycoprotein, about 1 to 10 kd in size, comprised of approximately 95.5% carbohydrate and 4.5% protein with the activity contained in the carbohydrate region of the compound.

The extract is produced by a process comprising removing the cartilage of the cartilaginous fish or its embryo, grinding the cartilage to release the chondrocytes, culturing the chondrocytes, and isolating the anti-angiogenic extract from the cultured chondrocytes and/or the chondrocyte media. In one aspect, the chondrocytes are cultured using the specialized growth media containing the shark serum.

This invention also provides a method for inhibiting the growth of endothelial cells by delivering to the cells a growth inhibitory amount of the extract. This invention also provides a method of inhibiting vascularization in a tissue by delivering

to the tissue an anti-vascularization amount of the extract. Methods of treating various diseases, including cancer and arthritis, are also provided herein.

The present invention also provides a method for culturing chondrocytes does not require an artificial matrix to promote attachment and growth of embryonic chondrocytes. The chondrocytes are able to attach and grow to a confluent level in approximately one week. This result is unexpected since the chondrocytes from adult cartilaginous fish, e.g., sharks failed to attach without the aid of an artificial matrix, and had a doubling time of 60 days with the use of an artificial matrix, after which the growth rate declined and new agarose was needed (see O'Hara (1995) supra, p. 49).

Further, a specialized growth media containing the fish serum has been developed. The serum is filtered from the blood and contains the chondrocytes' natural nutrients and hormones needed for optimal growth, including a parathyroid hormone related protein (PTHrp) which plays a major role in chondrocyte proliferation and differentiation (see Gelsleichter, J. and Musick, J.A., Abstract and Presentation at the July 17, 1998 American Elasmobranch Society Conference, University of Guelph, Guelph Canada, see also Gelschichter, J. & Musick, J.A. (1999) J. Exp. Zoo. **284**:549-556).

The use of embryonic cartilaginous cartilage as a source for chondrocytes instead of adult cartilage has further benefits. First, whereas the adult cartilage requires collagenase for dissociation (see O'Hara (1995) supra, p.21), no enzyme is needed to dissociate the embryonic cartilage. This reduces the risks of breaking down the integrity of the chondrocytes and contaminating the culture.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram showing a method for preparing an anti-angiogenic extract from embryonic shark cartilage.

FIG. 2 is a schematic diagram showing a method for preparing the shark serum.

FIGS. 3A and 3B are schematic diagrams showing a method for isolating the angiogenesis inhibitor from the chondrocytes and chondrocyte media.

MODES FOR CARRYING OUT THE INVENTION

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature.

Definitions

As used herein, certain terms may have the following defined meanings.

As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

All numerical designations, e.g., pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied (+) or (-) by increments of 0.1. It is to be understood, although not always explicitly stated that all numerical designations are preceded by the term "about". It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are well known in the art.

The term "isolated" means separated from constituents, cellular and otherwise, in which the compound is normally associated with in nature.

The term "medium" and "media" , used interchangeable and in either the singular or plural form, refer to the composition of various substance required for the growth of the cells.

A "subject" or "host" is a vertebrate, preferably an animal or mammal, more preferably a human patient. Mammals include, but are not limited to, murines, simians, human patients, farm animals, sport animals, and pets.

The terms "cancer," "neoplasm," and "tumor," used interchangeably and in either the singular or plural form, refer to cells that have undergone a malignant transformation that makes them pathological to the host organism. Primary cancer cells (that is, cells obtained from near the site of malignant transformation) can be readily distinguished from non-cancerous cells by well-established techniques, particularly histological examination. The definition of a cancer cell, as used herein, includes not only a primary cancer cell, but also any cell derived from a cancer cell ancestor. This includes metastasized cancer cells, and *in vitro* cultures and cell lines derived from cancer cells. When referring to a type of cancer that normally manifests as a solid tumor, a "clinically detectable" tumor is one that is detectable on the basis of tumor mass; e.g., by such procedures as CAT scan, magnetic resonance imaging (MRI), X-ray, ultrasound or palpation. Biochemical or immunologic findings alone may be insufficient to meet this definition.

As used herein, "inhibit" means to delay, slow, or stop the growth, proliferation or cell division of endothelial cells or the formation of blood vessels in tissue. Methods to monitor inhibition include, but are not limited to endothelial cell proliferation assays, measurement of the volume of a vascular bed by determination of blood content and quantitative determination of the density of vascular structures. When the culture is a mixture of cells, neovasculanization is monitored by quantitative measurement of cells expressing endothelial cell specific markers such as angiogenic factors, proteolytic enzymes and endothelial cell specific cell adhesion molecules.

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin REMINGTON'S PHARM. Sd., 15th Ed. (Mack Publ. Co., Easton (1975)).

An "effective amount" is an amount sufficient to effect beneficial or desired results. For example, a therapeutic amount is one that achieves the desired therapeutic effect. This amount may be the same or different from a prophylatically effective amount, which is an amount necessary to prevent onset of disease or disease symptoms. An effective amount can be administered in one or more administrations, applications or dosages.

The term "cartilaginous fish" or "fish" as used in reference to any of the elasmobranch commonly known as shark, ray and skate.

The term "adult" as used in reference to a shark, or other cartilaginous fish includes a shark or fish that has been naturally born, or hatched in the case of an egg laying species, i.e. is no longer in a gestational stage.

The term "chondrocyte" refers to a cartilage cell.

The term "cartilage" refers to the entire endoskeleton of a cartilaginous fish.

The term "anti-angiogenic" includes anti-inflammatory properties.

The present invention provides an anti-angiogenic and anti-inflammatory extract and method of producing the extract. The extract can be isolated from cultured chondrocytes derived from the cartilage of a cartilaginous fish and from the chondrocyte culture medium. Also provided is a specialized growth medium useful for culturing the cells which produce the new extract. The extract contains the biologically active agent which comprises a proteoglycan or glycoprotein, about 1 to 10 kd in size, comprised of approximately 95.5% carbohydrate and 4.5% protein with the activity contained in the carbohydrate region of the compound. Alternatively the biologically active glycoprotein is about 10 kd in size.

Also provided is a process for producing the extract. It can be obtained from chondrocytes released from the cartilage of the embryonic shark, or alternatively the chondrocytes are cultured in a culture medium. The biologically active ingredient is secreted by the chondrocytes into the medium, and thus, can be isolated from the culture medium as well as from the cultured cells. In one aspect, the cells are cultured using a growth media which includes serum derived from the blood of a cartilaginous fish.

By way of example only, the fraction is separated by centrifuging the medium to retain a supernatant, filter sterilizing the supernatant, and fractionating the supernatant to isolate the fraction having a molecular weight from between about 1 and about 10 kd.

In one aspect, the process of this invention requires removing the cartilage of the cartilaginous fish or fish embryo, grinding the cartilage to release the chondrocytes, culturing the chondrocytes, and isolating the anti-angiogenic extract from the cultured chondrocytes and/or the chondrocyte medium. In one aspect, the chondrocytes are cultured using the specialized growth medium containing the shark serum to produce the extract.

Alternatively, the extract is isolated from the cartilage of an embryonic cartilaginous fish by removing the cartilage from a cartilaginous fish embryo, grinding said cartilage to release its chondrocytes, producing a paste containing the released chondrocytes, and culturing said chondrocytes by mixing the chondrocyte paste into a culture medium and placing the mixture in a tissue culture plate.

In a further aspect, the process requires culturing said chondrocytes by mixing the chondrocyte paste into a culture medium and placing the chondrocyte/medium mixture in a tissue culture plate.

The culturing step can further comprise incubating said chondrocyte/medium mixture in a CO_2 rich environment to enhance chondrocyte attachment, removing said chondrocyte/medium mixture from the incubation environment, and removing old culture medium and adding fresh culture medium as necessary for chondrocyte growth.

In one aspect, the incubating step includes placing said chondrocyte/medium mixture in an environment comprising about 5% CO_2 having a temperature of about

37°C, and leaving said mixture in said environment for about 24 hours to about 48 hours.

In yet a further embodiment, the culture medium includes serum derived from the blood of a cartilaginous fish.

In another aspect, the process further comprises releasing the chondrocyte/medium mixture from the plate, and re-culturing the chondrocytes.

In yet another aspect, the process comprises releasing said chondrocyte/medium mixture from the plate, and centrifuging said mixture to separate the culture medium from the chondrocytes.

In one aspect, the process further comprises storing the chondrocytes by deep freezing. The deep freezing can comprise the steps of mixing the chondrocytes with freezing media and freezing at about -80°C to about -90°C for about 1 day, and storing the chondrocyte and freezing medium mixture in liquid nitrogen for long term storage. The chondrocytes can be mixed with freezing medium containing serum derived for the blood of a cartilaginous fish.

The invention further provides a medium for culturing cartilaginous fish chondrocytes comprising serum derived from the blood of a cartilaginous fish. The medium is obtained from an embryo of a cartilaginous fish. In one aspect, the serum and chondrocytes are derived from the same species of cartilaginous fish, e.g., the serum and chondrocytes are derived from a shark embryo.

The serum can be prepared by filtering said blood to isolate lymph fluids and remove particles, centrifuging said lymph fluids and retaining the supernatant, and heating the supernatant to inactivate enzymes therein. An effective amount of trimethylamine N-Oxide, and glutamine can also be added to the medium.

This invention also provides a method for inhibiting the growth of endothelial cells by delivering to the cells a growth inhibitory amount of the extract. This invention also provides a method of inhibiting vascularization in a tissue by delivering to the tissue an anti-vascularization amount of the extract. The active ingredient can be administered to the subject by oral administration, intravenous, intra-peritoneal, or trans-dermal. The extract can be processed and can be administered in liquid or powdered form.

In one aspect, the subject is an animal, e.g., the animal is selected from the group consisting of a pet, a farm animal or a human patient.

This invention further provides a method of treating a disorder associated with pathological neovascularization in a subject, comprising administering to a subject a therapeutically effective amount of the extract described herein. Such disorders include but are not limited to a disorder is selected from the group consisting of cancer, arthritic conditions, neovascular-based dermatological conditions, diabetic retinopathy, Kaposi's Sarcoma, age-related macular degeneration, restenosis, telangectasia, glaucoma, keloids, corneal graft rejection, wound granularization, angiofibroma, Osler-Webber Syndrome, myocardial angiogenesis, and scleroderma. Alternatively, inhibiting angiogenesis prevents or inhibits fetal development of a fetus carried by the mammal.

The development of rheumatoid arthritis has been directly linked to the onset of angiogenesis in cartilage, which directly causes its degradation. Work done by Walsh (1998) Rheumatology 38(2):103-112, has shown that pathological angiogenesis plays a central role in the development of rheumatoid arthritis. It is hypothesized that the dosing of an effective amount of angiogenesis inhibitor to the affected area could lead to a decrease in arthritic symptoms and possibly the complete reversal of the conditions of rheumatoid arthritis.

It is thought that both diabetic retinopathy and aged-ralated macular degeneration can be treated effectively by the use of angiogenesis inhibitors. It is known that diabetic retinopathy is caused by the aggressive growth of blood vessels in the retina, leading to permanent blindness. Aged-related macular degeneration is also attributed to aggressive angiogenesis, also leading to permanent vision loss. It is predicted that the use of an angiogenesis inhibitor to prevent the onset and continuation of angiogenesis would be an extremely efficient method of treatment for these two conditions.

The symptoms of psoriasis have been positively linked to the onset of increased angiogenesis in affected individuals. It is hypothesized that the addition of an effective quantity of angiogenesis inhibitors will negate the effects of this abnormal angiogenesis, thereby preventing the occurrence of psoriasis symptoms.

Further provided is a method for screening for a therapeutic agent for inhibiting neovascularization, comprising contacting the agent with a suitable cell or tissue sample; contacting a separate sample of the suitable cell or tissue sample with a therapeutically effective amount of the extract of this invention; and comparing the

growth of the sample of step (a) with the growth of the sample of step (b), and wherein any agent of step (a) that inhibits the growth to the same or similar extent as the sample of step (b) is a therapeutic agent for inhibiting neovascularization or the growth of endothelial cells. The contacting is in vitro or in vivo.

Yet further provided is a kit for treating a disorder associated with pathological neovascularization in a host, comprising a therapeutically effective amount of the extract as described herein and instructions for use.

Materials and Methods

Method of preparation

FIG. 1 is a schematic diagram showing a method of preparing an anti-angiogenic extract from embryonic shark cartilage. This procedure includes: 1) removing the cartilage of a shark embryo, 2) grinding the cartilage to release the chondrocytes, 3) culturing the chondrocytes, preferably using a specialized growth media containing shark serum, and 4) isolating the anti-angiogenic extract from the cultured chondrocytes and chondrocyte culture media.

Sharks are members of the *Chondrichthyes* family, or cartilaginous fishes, meaning that their endoskeleton is composed entirely of cartilage. Although this application refers to sharks as the main source for the chondrocytes, it is believed that any cartilaginous fish including sharks, rays and skates may be used. The Spiny Dogfish, Swell shark, and Thresher shark, which are all members of the Elasmobranch family, are particularly good sources for embryonic chondrocytes, due to availability and ease of acquiring these sharks.

Once the embryo's endoskeleton is developed it is a potential source of chondrocytes. The preferred time period for use depends on the species. The pregnancy stage for using the Spiny Dogfish embryo is preferably from 4 to 12 months, since by the fourth month the embryos will have developed an endoskeleton. The number of embryos for this shark is variable, ranging from 1 to 10 per shark. Development for a Swell shark embryo, which is an egg laying species, is approximately 7.5 to 10 months, depending on the water temperature.

In the case of live bearing sharks, such as the Spiny Dogfish, the embryo is removed from the pregnant shark directly, preferably under sterile conditions in a hood. Unfortunately, current removal procedures result in the death of the mother,

mainly for humane purposes. Where the shark is an egg-layer, such as Swell, the embryo can be acquired by opening the egg and without sacrificing the mother.

Once the shark embryo is obtained, its endoskeleton is removed neatly and the surrounding flesh, skin, and organs are cleaned away. The endoskeleton is first roughly cleaned and then transferred to a sterile hood for further trimming and cleaning.

Method No. 1.

The cleaned embryonic endoskeleton is then stored in a solution comprising Hepes/NaOH, Glucose, NaCl, KCl, Na_2HPO_4, Urea and Trimethylamine-N-oxide. A preferred storage solution (referred to as "solution B") consists approximately of 11.92 g Hepes/NaOH pH 7.6, 7.1g Glucose, 15.84g NaCl, 0.22g KCL, 0.81g Na_2HPO_4, 20g Urea, and 6g Trimethylamine-N-oxide. Such a solution is used to prevent dehydration due to the high salt concentration in the shark's blood from living in a high salt environment. Trimethylamine-N-oxide is believed to allow the shark's enzymes to continue to function in this high salt environment (Grogan and Lund (1990) supra).

The embryonic endoskeleton is removed from the storage solution and rinsed with clean solution B. The moist endoskeleton is then chopped finely by manually dicing with a knife followed by grinding to a paste of fine granules of particulate matter, using a sterile mortar and pestle. This releases the chondrocytes of the embryonic endoskeleton.

The chondrocytes are suspended in about 5 ml of modified Optimum Minimum Essential Media (Opti-Mem) 20E media, preferably containing a specialized growth media prepared from shark serum (described below). The chondrocytes are mixed into the media using a pipette. In a preferred composition the modified Opti-Mem 20E media comprises, per 500 ml of media, approximately: 20 ml of 10X Opti-Mem (available through Gibco BRL); 1.99 g of Urea; 14.02g of NaCl; 2 ml of penicillin and streptomycin, both at 500 units/ml; 2 ml of 7.5% $NaHCO_3$ (which results in a pH = 7.2); the specialized growth media containing 1.4 g of trimethylamine-N-oxide (TMAO), 2ml of 200 mM Glutamine, and 5 to 20 ml of heat inactivated shark serum; and double distilled water ("dd H_2O") to bring the solution to 500 ml. All ingredients, except for the shark serum, are commercially

available through companies such as Gibco BRL and Sigma, and are preferably filter sterilized and stored in sterile tissue culture grade glassware.

The heat inactivated shark serum in the specialized growth media supplies the chondrocytes with nutrients for 2-3 days of growth. TMAO is used to simulate normal growth conditions in an aqueous high salt environment (see Grogan and Lund (1990) supra), and the glutamine is an essential amino acid that is commonly used in growth media and facilitates improved growth of embryonic chondrocytes. The shark serum is composed of the lymph fluids (liquid filtrate) filtered from shark blood. FIG. 2 is a schematic diagram showing a method for preparing the shark serum. Millipore filters, approximately 100 nm pore size were used to filter the shark's blood. The purpose of filtering is to remove all bacterial and fungal particles, and mycoplasmas, and to lower the hemoglobin and endotoxin amounts, as these all affect the growth medium. It may be possible to also use a smaller size filter, perhaps as low as 50 nm, to further filter out additional viral particles from the blood. After filtering, the liquid filtrate is centrifuged to provide the liquid supernatant, which comprises the serum. The liquid supernatant is then heated to inactivate the enzymes, thus producing a heat inactivated serum. The amount of heat required may vary, depending on the type of shark. According to a preferred procedure, the supernatant is heated at about 68 °C for approximately 30 minutes in the case of thresher shark.

Using shark serum is believed to promote faster growth compared to conventional fetal bovine serum used by O'Hara (see O'Hara (1995) supra p. 101) since it contains the shark chondrocytes' natural nutrients and hormones needed for optimum growth. Shark serum also contains a parathyroid hormone related protein (PTHrp), which is a hormone that plays a major role in chondrocyte proliferation and differentiation (see Gelsleichter, J. and Musick J.A., Abstract and Presentation at the July 17, 1998 American Elasmobranch Society Conference, supra; and Gelsleichter, J. & Musick, J.A. (1999) supra). It can also be beneficial to use embryo shark blood for the serum in addition to or instead of adult shark blood since it more closely resembles the natural environment of the embryonic chondrocytes, and can contain nutrients, which are more suitable for their growth.

The modified media and paste containing chondrocytes is placed in wells on a Corning Tissue Culture 24-well plate and then incubated in approximately 5% CO_2 at 37 °C for a period of 24-48 hours until chondrocyte attachment. Old media is removed

via vacuum and new media is added when necessary (usually every 2-3 days depending on the amount of growth). The amount added depends on the size of the culture, (approximately 0.5ml per well for a tissue culture plate, or from 3-5 ml on a tissue culture flask). The growth is regularly checked for confluence (meaning that the plate is fully covered in cells when viewed through a scanning microscope). The growth reaches Confluence approximately 1 week following the initial culturing step of mixing the chondrocytes with the medium. Upon confluence (approximately 10^8cells/ml for a plate) trypsin is added to detach the cells from the bottom of the plate. The cells can then be re-cultured, split, stored, or recovered for use, analysis, or further extraction and isolation of the angiogenesis inhibitor.

To recover after culturing and trypsination, the chondrocytes are concentrated by spinning down in a centrifuge (preferably at a centrifuge setting of 1,000 rpm for 5 minutes at 4 °C), and the solid material is re-suspended in water. The re-suspended material is then lyophilized to remove the greater majority of the water and to freeze dry the chondrocytes, after which the material is re-hydrated as desired. An alternate procedure for use, analysis, or further extraction and isolation of the anti-angiogenic component will be discussed below.

To re-culture after trypsination, the cells are incubated for 5 minutes, preferably in 5% CO_2 at 37 °C, and are split onto different tissue culture plates. Additional media is added to again promote growth. This process may be repeated as necessary.

Theoretically, an unlimited amount of chondrocyte cells can be cultured from a single sample of embryonic shark cartilage, but this is dependent upon the sturdiness of the cells isolated and grown from that sample. Some cell lines are much sturdier and stronger than others.

If a successful cell line is developed, a sample can be deep frozen at –80° C and stored for extended periods of time while the remaining cells continue to be grown and used. Deep-freezing the cells also insures that a supply of embryonic chondrocytes will be available in case of emergency.

To deep freeze the cells, a stock supply of embryonic chondrocytes is taken from the growth media at a confluent level by trypsination, and is centrifuged and mixed with freezing media. The mixture is then frozen and placed at approximately -80 to –90 °C for long-term storage. The freezing media contains approximately 10%

Dimethyl Sulfoxide (DMSO) and 90% shark serum. Shark serum is believed to be beneficial when re-culturing the frozen cells. The entire freezing process must be done on ice (~0 °C) to keep the cells viable.

To prevent contamination and to insure that any of the activity of the cultured chondrocytes does not degrade due to mishandling, it is preferable to store any intermediate product at 4 °C as soon as possible. Additionally, to prevent contamination, the entire process should be kept as sterile as possible.

Isolation of the angiogenesis inhibitor

The angiogenesis inhibitor is present in both the isolated chondrocytes and culture medium, as the chondrocytes also secrete an angiogenic inhibitor into the medium. Upon filtration and isolation, the inhibitor is present in the 1-10 kd fraction with a molecular weight of approximately 10 kd. The inhibitor comprises a heat stable proteoglycan or glycoprotein, comprised of approximately 95.5% carbohydrate and 4.5% protein, with keratan sulfate comprising the carbohydrate part, and peptide units. Additionally, it is likely that an N-glycosidic bond links the carbohydrate and peptide. The effectiveness of the inhibitor is also believed to be concentration dependent and sensitive to extreme pH conditions.

FIG. 3A is a schematic diagram showing a method for isolating the inhibitor from the chondrocytes after separation from the culture media by trypsination and centrifugation (see FIG.1). First, the isolated chondrocytes are lyzed in order to release their content. This is done by suspending the cells in approximately 0.5%-1% Triton X-100 in Phosphate buffered saline (PBS) (Sigma), then placing the solution on ice for 5 to 15 minutes, and again centrifuging (at 14k for 2 min on a tabletop centrifuge) to remove debris from the liquid extract. The liquid extract is then fractionated, using a Millipore membrane, to isolate the fraction between 1 and 10 kd.

FIG. 3B is a schematic diagram showing a method for isolation of the inhibitor from the media, which is retained after trypsination and centrifugation to separate the media from the cultured chondrocytes (see FIG.1). First, the media is again centrifuged (10k for about 30 min. at about 4 °C) to pellet out coagulants. The media is then filter sterilized using a sterile 0.2 micron filter, and fractionated using a Millipore membrane, to isolate the fraction between about 1 and about 10 kd.

The 10 kd component is further isolated from the 1-10 kd fraction by gel filtration chromatography. According to a preferred method, Sephacryl S-300 HR (Sigma) is packed to a height of 30 cm in a glass column 1.5 cm in diameter. The column is equilibrated with a buffer such as PBS or dd H_2O. The sample, 1 mg of the 1-10 kd sample dissolved in 2 ml of the same buffer, is applied to the column. Elution is achieved with the same buffer at a rate of 15 ml per hour, and 2 ml fractions are collected. The column is calibrated using Gel Filtration Molecular Weight Markers (Sigma). Guanidine hydrochloride (GuHCl) (~6M) may also be used as a buffer. In this case, the collected fractions are dialyzed (1,000 MW, Spectra 7) against double distilled H_2O to remove the salt.

It is believed that aggregation, which is the interaction of the molecules with one another to form multi-molecular clusters, impedes separation by fractionation and gel filtration as described. Fractionation performed to isolate the 1-10 kd fraction will result in loss of some molecules in that range since these molecules will form aggregates above that range. Isolation of the 10 kd component will similarly result in loss of aggregated molecules. The use of GuHCl in gel filtration helps to dissociate these aggregates. It may also be possible to use GuHCl in the initial fractionation of the 1-10 kd molecules to reduce aggregation. However, GuHCl is believed to be harsh and degrade the compound. Therefore, H_2O and PBS are the preferred buffers.

Comparison between method of culturing embryonic shark chondrocytes vs. adult shark chondrocytes

Published literature teaches culturing chondrocytes from the cartilage of adult sharks. This requires an artificial matrix for aiding the attachment and infiltration of the chondrocytes before the chondrocytes begin to grow. (see O'Hara (1995) supra and O'Hara and Wong (1996) supra). Attachment of the cells with use of agarose, which is the preferred artificial matrix, according to O'Hara, takes from 2 to 3 weeks. The cells have a doubling time of approximately 60 days after which the growth rate declines (O'Hara (1995) p. 49 supra). Once growth rate declines the agarose containing the cells must be split and transferred to another plate containing fresh agarose. Additionally, use of the agarose makes it more difficult to release the cells (O'Hara (1995) p.53, supra).

On the other hand, the embryonic chondrocytes developed according to the present invention do not require an artificial matrix to enable them to adhere to the walls of the tissue culture, and have a significantly faster growth rate (approximately 1 week) compared to the adult shark chondrocytes. This is believed to be because embryonic cartilage is still in the process of growing and developing. Hence, it still contains much of the enzymes, growth hormones, and other signaling factors that are essential for healthy growth and development. The adult cartilage, however, does not contain as high or as varied an amount of factors as it is no longer in a state of growth, but rather in an equilibrium, or else a gradual degradation state. Additionally, use of the specialized growth media comprising shark serum is believed to further increase the growth rate of the chondrocytes.

Moreover, since the cartilage of adult sharks is very brittle and physically hard, the processing procedure for this material can result in the integrity of the chondrocytes breaking down. The cartilage of adult sharks must be ground-up by first chopping with a knife and then using a blender, rather then a mortar and pestle. The ground up cartilage must then be dissociated to soften and degrade the cartilage to the point that it is possible to cultivate individual cells from the cartilage. To achieve this, a solution consisting of collagenase (see O'Hara (1995) p.21 supra) is added to the cartilage. The cartilage is soaked in the solution for approximately 30 minutes to 12 hours, depending on the size of the cartilage, to allow for dissociation. The solution is then centrifuged to pellet the cells, and the dissociation solution completely removed before re-suspending the retained pellets in the modified Opti-Mem 20E media.

There are several problems with having to use a disassociation agent such as collagenase. First, it risks breaking down the integrity of the chondrocytes and increases the risk of contamination. There is also a risk of over dissociation if the cartilage is left too long in the solution. Additionally, it is more costly to develop and requires additional equipment, chemicals, and procedural steps. Moreover, adult cartilage is harder to initially clean.

In contrast, nothing is needed to dissociate the embryonic cartilage, since it is already extremely malleable. Simply cutting and grinding using a mortar and pestle is adequate to release the chondrocytes.

Treatment of a tumored mouse using the prior art inhibitor

The isolated 1-10 kd fraction of shark cartilage extract, derived from adult Thresher shark according to the method described in Liang's (1997), supra, was tested on a mouse having a noticeable tumor on its neck which was initially approximately 3 cm in diameter. The mouse was monitored for a period of over 3 months in which the mouse was orally fed water containing 1 mg/ml of the extract.

Three weeks after initial administration of 4 ml of the 1 mg/ml solution, the tumor started shrinking. After 4 weeks, the mouse showed noticeable improvement in movement and activity, and the tumor turned black and continued shrinking. After 8 weeks, the tumor had shrunk to less then half its initial size (approximately 1.5 cm in diameter). After 10 weeks the tumor had shrunk to 0.5 cm in size, and the mouse showed significant improvement in movement and activity. At the end of the study, which was 13 weeks, the tumor showed continued shrinkage and the mouse remained extremely active.

The results of this study are summarized in the table below:

Time Period of treatment with Shark Cartilage Extract (weeks)	Tumor Size (cm in diameter)
0	~3
5	~1.5
10	~0.5

Additional experiments also performed on mice showed an increase in the life span of the mice by a minimum of 12% with administration of the adult shark chondrocyte extract.

The embryonic shark derived agents can be used where prior materials were used. However, the new materials are much more efficient and practical to produce, and are believed to have inhibitory properties which are at least equal, if not superior to the prior materials.

The inhibitor may be administered in different forms including solution form, such as intravenously, and powdered form. Potential conditions for treatment by angiogenesis inhibition include cancer, solid tumors, rheumatoid arthritis, osteoarthritis, psoriasis, diabetic retinopathy, age related macular degeneration, restinosis, Kaposi's sarcoma, leukemia, metastasis, benign tumors, Osler-Webber

syndrome, myocardial angiogenesis, plaque neovascularzation, telangeictasia, hemophilic joints, angiofibroma, wound granulation, glaucoma, corneal graft rejection, atherosclerosis, scleroderma, and keloids. Additionally, anti-angiogenesis can be used to treat anti-inflammatory, anti- collagenolytic, in vivo anti-tumor proliferating and direct in vitro anti-tumor proliferating activities. (See DuPont U.S. Pat No. 6,028,118). There is also a potential use for anti-angiogenesis as a birth control since fetal development is driven by angiogenesis (See Klauber N., et al. (1997) "Critical Components of the Female Reproductive Pathway are Suppressed by the Angiogenesis Inhibitor" AGM-1470 Nat Med. 3(4):443-446).

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications will be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

CLAIMS

1. A method of inhibiting angiogenesis or treating inflammation responses in mammals comprising delivering to the mammal an effective amount of a biologically active extract isolated from the cartilage of an embryonic cartilaginous fish.

2. The method of claim 1, wherein inhibiting angiogenesis prevents or inhibits fetal development of a fetus carried by the mammal.

3. The method of claim 1, wherein said cartilaginous fish is a shark.

4. The method of claim 1, wherein said biologically active extract comprises glycoproteins or proteoglycans having a molecular weight of about 1 kd to about 10 kd..

5. The method of claim 4, wherein said glycoproteins or proteoglycans have a molecular weight of about 10 kd.

6. The method of claim 1, wherein said biologically active extract is isolated from chondrocytes released from the cartilage of an embryonic shark.

7. The method of claim 6, wherein said chondrocytes are cultured in a culture medium.

8. The method of claim 7, wherein said biologically active extract is isolated from said medium.

9. The method of claim 1, wherein said biologically active extract is isolated by removing the cartilage from said embryo and releasing chondrocytes from said cartilage.

10. The method of claim 9, further comprising culturing said chondrocytes.

11. The method of claim 10, further comprising isolating said biologically active extract from said chondrocytes.

12. The method of claim 10, further comprising isolating said biologically active extract from a culture medium.

13. The method of claim 10, wherein said culturing step further comprises using a growth medium which includes serum derived from the blood of a cartilaginous fish.

14. The method of claim 1, wherein said biologically active extract is administered in liquid form.

15. The method of claim 1, wherein said biologically active extract is administered in powdered form.

16. A biologically active compound having an apparent molecular weight from about 1kd to 10kd, that inhibits angiogenesis or treats inflammation responses in mammals, said biologically active compound is isolated from the cartilage of an embryonic cartilaginous fish.

17. The compound of claim 16 prepared by:

a) removing the cartilage from a cartilaginous fish embryo,

b) grinding said cartilage to release its chondrocytes, producing a paste containing the released chondrocytes, and

c) culturing said chondrocytes by mixing the chondrocyte paste into a culture medium and placing the mixture in a tissue culture plate.

18. The compound of claim 16 having a molecular weight of about 10 kd.

19. The compound of claim 16, wherein said compound is a proteoglycan or glycoprotein.

20. The compound of claim 16, wherein said compound is about 4.5% protein.

21. A method of preparing an biologically active extract for use in the treatment of angiogenesis or inflammation responses in mammals comprising the steps of:

a) removing cartilage from a cartilaginous fish embryo,

b) grinding said cartilage to release chondrocytes, producing a paste containing the released chondrocytes, and

c) culturing said chondrocytes by mixing the chondrocyte paste into a culture medium and placing the chondrocyte/medium mixture in a tissue culture plate.

22. The method of claim 21, wherein said culturing step further comprises:

a) incubating said chondrocyte/medium mixture in a CO_2 rich environment to enhance chondrocyte attachment,

b) removing said chondrocyte/medium mixture from the incubation environment, and

c) removing old culture medium and adding fresh culture medium as necessary for chondrocyte growth.

23. The method of claim 22, wherein said incubating step includes:

a) placing said chondrocyte/media mixture in an environment comprising 5% CO_2 having a temperature of 37°C, and

b) leaving said mixture in said environment for about 24 hours to about 48 hours.

24. The method of claim 21, wherein said culture medium includes serum derived from the blood of a cartilaginous fish.

25. The method of claim 21, further comprising:

a) releasing the chondrocyte/medium mixture from the plate, and

b) re-culturing the chondrocytes.

26. The method of claim 21, further comprising:

a) releasing said chondrocyte/medium mixture from the plate, and

b) centrifuging said mixture to separate the culture medium from the chondrocytes.

27. The method of claim 26, further comprising storing the chondrocytes by deep freezing.

28. The method of claim 27, wherein deep freezing comprises:

a) mixing the chondrocytes with freezing medium and freezing at about -80°C to about -90°C for about 1 day, and

b) storing the chondrocyte and freezing medium mixture in liquid nitrogen for long term storage.

29. The method of claim 27, wherein the chondrocytes are mixed with freezing medium containing serum derived for the blood of a cartilaginous fish.

30. The method of claim 26, further comprising separating out a fraction having a molecular weight from about 1 kd to about 10 kd from the chondrocytes.

31. The method of claim 30, wherein said fraction is separated by:

a) lysing the chondrocytes to release their content, and

b) fractionating said chondrocytes to isolate the 1 to 10 kd fraction.

32. The method of claim 30, further comprising separating the components having a molecular weight of about 10 kd from said fraction by gel filtration.

33. The method of claim 26, further comprising separating out a fraction having a molecular weight from about 1 kd to about 10 kd from the medium.

34. The method of claim 33, wherein said fraction is separated by:

a) centrifuging the media to retain a supernatant,

b) filter sterilizing the supernatant, and

c) fractionating the supernatant to isolate the fraction having a molecular weight from between about 1 and about 10 kd.

35. The method of claim 34, further comprising separating the components having a molecular weight of about 10 kd from said fraction by gel filtration.

36. A media for culturing cartilaginous fish chondrocytes, said medium comprising serum derived from the blood of a cartilaginous fish.

37. The media of claim 36, wherein the serum is obtained from an embryo of a cartilaginous fish.

38. The medium of claim 36, wherein the serum and chondrocytes are derived from the same species of cartilaginous fish.

39. The medium of claim 36, wherein the serum and chondrocytes are derived from a shark embryo.

40. The medium of claim 36, said serum prepared by:

a) filtering said blood to isolate lymph fluids and remove particles,

b) centrifuging said lymph fluids and retaining the supernatant, and

c) heating the supernatant to inactivate enzymes therein.

41. The medium of claim 36, further comprising:

a) trimethylamine N-Oxide, and

b) glutamine.

42. A method of treating a disorder associated with pathological neovascularization in a subject, comprising administering to a subject a therapeutically effective amount of the compound of claim 16.

43. A method of claim 42, wherein the disorder is selected from the group consisting of cancer, arthritic conditions, neovascular-based dermatological conditions, diabetic retinopathy, Kaposi's Sarcoma, age-related macular degeneration, restenosis, telangectasia, glaucoma, keloids, corneal graft rejection, wound granularization, angiofibroma, Osler-Webber Syndrome, myocardial angiogenesis, and scleroderma.

44. The method of claim 43, wherein the disorder is an arthritic condition selected from the group consisting of rheumatoid arthritis and osteoarthritis.

45. The method of claim 42, wherein the delivering is by oral administration, intravenous, intra-peritoneal, or trans-dermal.

46. The method of claim 42, wherein the subject is an animal.

47. The method of claim 46, wherein the animal is selected from the group consisting of a pet, a farm animal or a human patient.

48. A method for screening for a therapeutic agent for inhibiting neovascularization, comprising:

a) Contacting the agent with a suitable cell or tissue sample;

b) Contacting a separate sample of the suitable cell or tissue sample with a therapeutically effective amount of the extract of claim 4; and

c) comparing the growth of the sample of step (a) with the growth of the sample of step (b), and wherein any agent of step (a) that inhibits the growth to the same or similar extent as the sample of step (b) is a therapeutic agent for inhibiting neovascularization or the growth of endothelial cells.

49. The method of claim 48, wherein the contacting is in vitro or in vivo.

50. A kit for treating a disorder associated with pathological neovascularization in a host, comprising a therapeutically effective amount of the compound of claim 16 and instructions for use.

51. The kit of claim 50, wherein the disorder is selected from the group consisting of cancer, arthritic conditions, neovascular-based dermatological conditions, diabetic retinopathy, Kaposi's Sarcoma, age-related macular degeneration, restenosis, telangectasia, glaucoma, keloids, corneal graft rejection, wound granularization, angiofibroma, Osler-Webber Syndrome, myocardial angiogenesis, and scleroderma.

52. The kit of claim 50, wherein the disorder is an arthritic condition selected from the group consisting of rheumatoid arthritis and osteoarthritis.

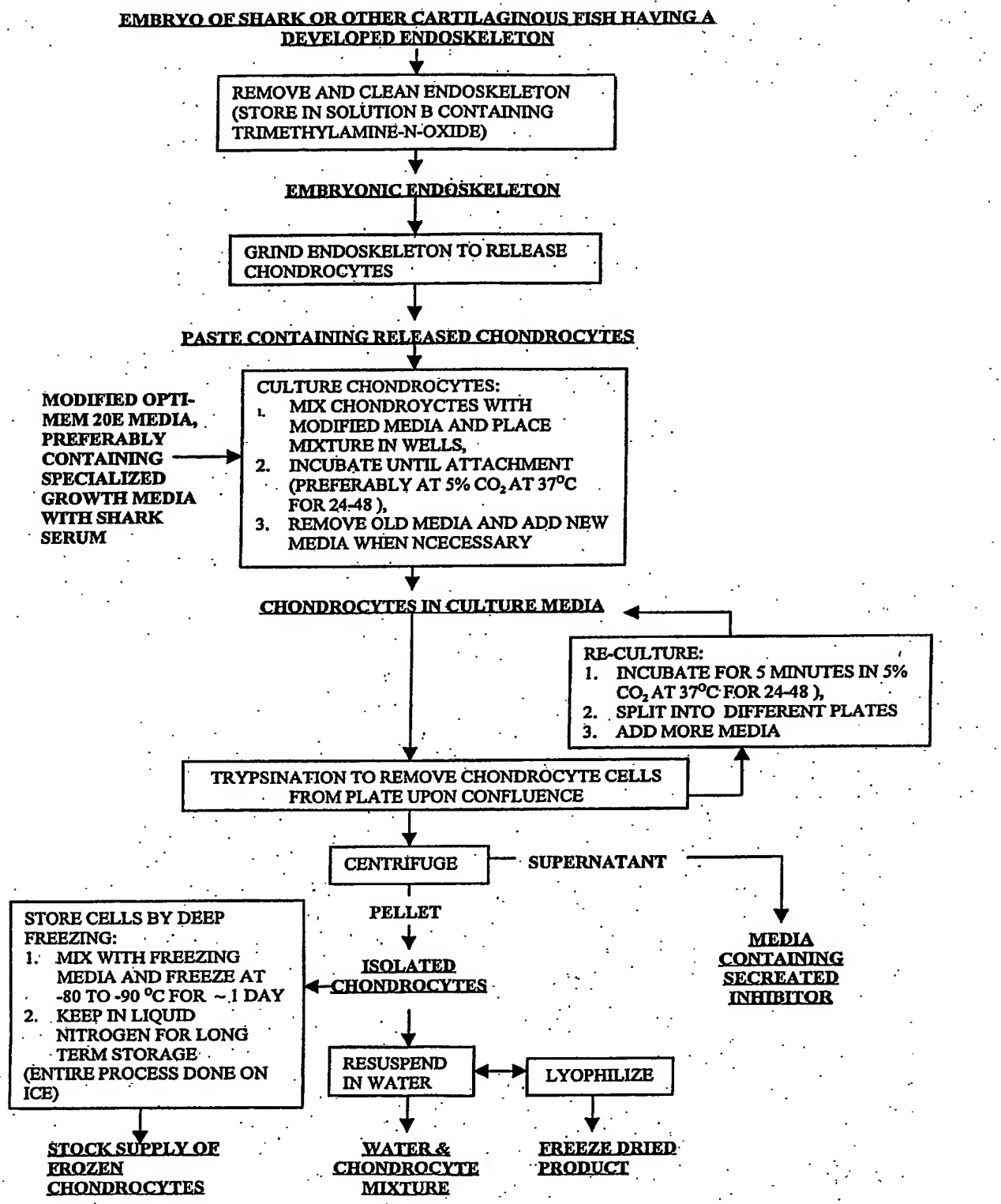
EMBRYO OF SHARK OR OTHER CARTILAGINOUS FISH HAVING A DEVELOPED ENDOSKELETON
REMOVE AND CLEAN ENDOSKELETON (STORE IN SOLUTION B CONTAINING TRIMETHYLAMINE-N-OXIDE)
EMBRYONIC ENDOSKELETON
GRIND ENDOSKELETON TO RELEASE CHONDROCYTES
PASTE CONTAINING RELEASED CHONDROCYTES
MODIFIED OPTI-MEM 20E MEDIA, PREFERABLY CONTAINING SPECIALIZED GROWTH MEDIA WITH SHARK SERUM
CULTURE CHONDROCYTES:
1. MIX CHONDROYCTES WITH MODIFIED MEDIA AND PLACE MIXTURE IN WELLS,
2. INCUBATE UNTIL ATTACHMENT (PREFERABLY AT 5% CO_2 AT 37°C FOR 24-48),
3. REMOVE OLD MEDIA AND ADD NEW MEDIA WHEN NCECESSARY
CHONDROCYTES IN CULTURE MEDIA
RE-CULTURE:
1. INCUBATE FOR 5 MINUTES IN 5% CO_2 AT 37°C FOR 24-48),
2. SPLIT INTO DIFFERENT PLATES
3. ADD MORE MEDIA
TRYPSINATION TO REMOVE CHONDROCYTE CELLS FROM PLATE UPON CONFLUENCE
CENTRIFUGE
SUPERNATANT
PELLET
MEDIA CONTAINING SECREATED INHIBITOR
ISOLATED CHONDROCYTES
STORE CELLS BY DEEP FREEZING:
1. MIX WITH FREEZING MEDIA AND FREEZE AT -80 TO -90 °C FOR ~1 DAY
2. KEEP IN LIQUID NITROGEN FOR LONG TERM STORAGE
(ENTIRE PROCESS DONE ON ICE)
RESUSPEND IN WATER
LYOPHILIZE
STOCK SUPPLY OF FROZEN CHONDROCYTES
WATER & CHONDROCYTE MIXTURE
FREEZE DRIED PRODUCT

FIG. 1

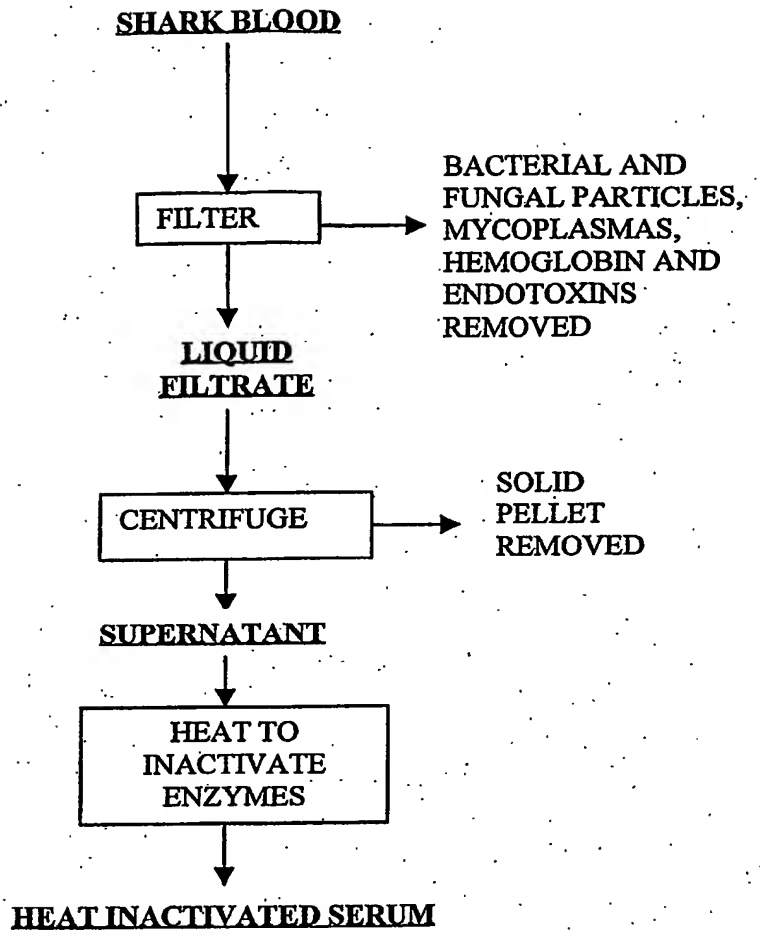
SHARK BLOOD
FILTER
BACTERIAL AND FUNGAL PARTICLES, MYCOPLASMAS, HEMOGLOBIN AND ENDOTOXINS REMOVED
LIQUID FILTRATE
CENTRIFUGE
SOLID PELLET REMOVED
SUPERNATANT
HEAT TO INACTIVATE ENZYMES
HEAT INACTIVATED SERUM

FIG. 2

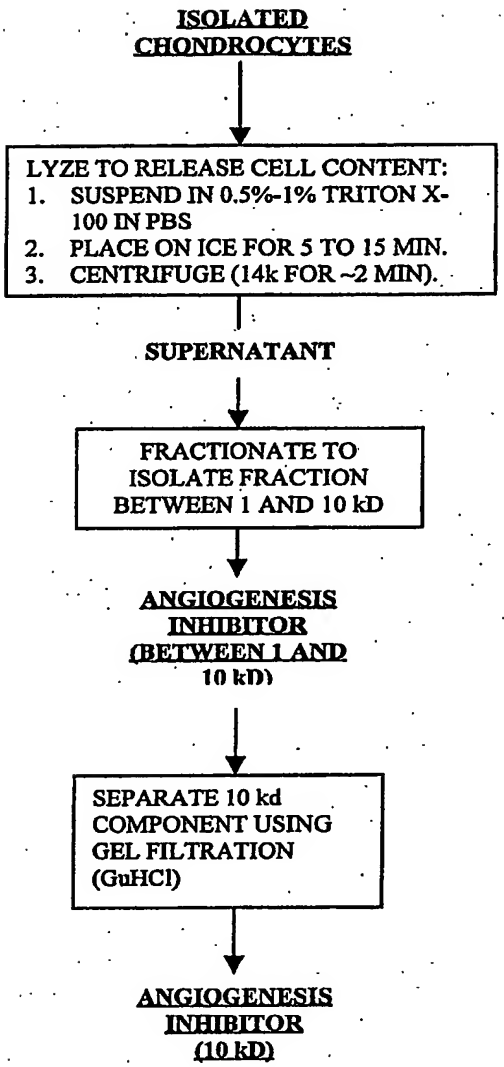
ISOLATED CHONDROCYTES
LYZE TO RELEASE CELL CONTENT:
1. SUSPEND IN 0.5%-1% TRITON X-100 IN PBS
2. PLACE ON ICE FOR 5 TO 15 MIN.
3. CENTRIFUGE (14k FOR ~2 MIN).
SUPERNATANT
FRACTIONATE TO ISOLATE FRACTION BETWEEN 1 AND 10 kD
ANGIOGENESIS INHIBITOR (BETWEEN 1 AND 10 kD)
SEPARATE 10 kd COMPONENT USING GEL FILTRATION (GuHCl)
ANGIOGENESIS INHIBITOR (10 kD)

FIG. 3a

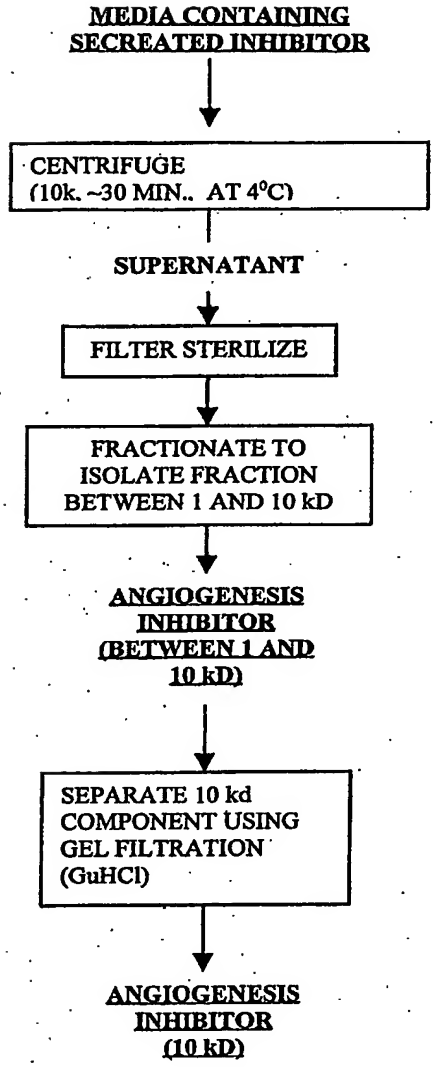
MEDIA CONTAINING SECREATED INHIBITOR
CENTRIFUGE (10k. ~30 MIN.. AT 4°C)
SUPERNATANT
FILTER STERILIZE
FRACTIONATE TO ISOLATE FRACTION BETWEEN 1 AND 10 kD
ANGIOGENESIS INHIBITOR (BETWEEN 1 AND 10 kD)
SEPARATE 10 kd COMPONENT USING GEL FILTRATION (GuHCl)
ANGIOGENESIS INHIBITOR (10 kD)

FIG. 3b